Original Article

Assesment of Protein Gene Product (Pgp9.5) Enzyme Activity Against Potential Peptide Substrate

Pgp9.5 Enzim Aktivitesinin Potansiyel Peptid Substratlarına Karşı Değerlendirilmesi

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Abstract

Objective. PGP9.5 is a human neuron specific ubiquitin carboxyl-terminal hydolase that has been shown by immunuhistochemistry to be present selectively in ubiquitinated inclusions in chronic human degenerative disease. Paraffin sections known to contain ubiquitin-protein conjugate immunureactivity in neurofibrillary tangles (NFT), cortical Lewy bodies, Rosenthal fibres and in Pick bodies were immunostained with PGP9.5. In Alzheimer's disease loosely arranged globose-type neurofibrillary tangles (NFTs) were immunostained together with neuritis surrounding senile plaques (SP). While PGP9.5 has been demonstrated to have ubiquitin carboxy-terminal ethyl esterase activity, there has not been clear identification of its substrate specificity. The main aim was, therefore, to purify PGP9.5 and study its carboxyl-terminal hydrolase activity using, as substrates, synthetic peptides that were chosen to reflect the known possible functions of the enzyme.

Materials and Methods. Ubiquitin is cleaved from conjugates by ubiquitin carboxyl-terminal hydrolases, one of which is protein gene product 9.5 (PGP 9.5). PGP9.5 was purified to homogeneity from human post-mortem brain tissue, its identity confirmed by protein sequence determination. Potential peptide substrates were incubated with PGP 9.5 and assayed by HPLC

Results. Overlap region of ubiquitin in branched gene products were not substrates. Evidence was obtained for cleavage of linearly-conjugated polyubiquitin.

Conclusion. Degradation of abnormal proteins by ubiquitin system depends on binding structure of ubiquitins. It has been shown that only linear ubiquitis are substrate for the PGP 9.5. The importance of it is not well understood.

Keywords: Protein gene product 9.5 (PGP9.5), Human brain, Synthetic substrates

Özet

Amaç. *PGP 9.5, kronik dejeneratif hastalıklarda özellikle ubiquitin ile işaretlenmiş inkluzyonlarda bulunan, immunohistokimyasal boyamalarla da gösterilen norona spesifik ubiquitin karboksi terminal hidroksilazdır. Demanslı hastaların post-mortem beyin kesitlerinde patolojik olarak görülen norofibriler düğümler, kortikal Lewy body, Rosenthal fiber ve Pick body'lerde PGP 9.5'in varlığı immunohistokimyasal boyama ile gösterildi. Alzheimer hastalığında yaygın olarak görülen norofibriler düğümler senil plakları çevreleyen nöronlar ile birlikte boyandı. PGP 9.5, ubiquitin karboksi terminal etil esteraz aktivitesi göstermişken, onun substrata spesifikliği açık değildir. Bu yüzden amacımız PGP 9.5'i pürifiye etmek ve enzimin hücre içinde yıkılacak proteinlere bağlanan ubiquitinlerin serbest hale gelmesindeki rolünü göstermektir.*

Gereç ve Yöntem. *Ubiquitin, ubiquitin karboksi terminal hidrolaz ile konjugatlarından ayrılır, bunlardan biri de PGP 9.5'tir. PGP 9.5 insan post-mortem beyin dokusunda homojenize edilip purifiye edildi. Protein sekansı yapılarak doğruluğu belirlendi. Potansiyel peptid substratlar PGP 9.5 ile inkube edildi ve HPLC ile analiz edildi.*

Bulgular. *Dallanmış poliubiquitin lerin PGP9.5 enzimi için substrat olmadığı fakat enzimin düz zincir şeklinde bağlanmış ubiquitinleri substrat olarak tanıdığı gösterilmiştir.*

Sonuç. *Hücre içindeki anormal proteinlerin ubiquitine bağımlı yıkımında ubiquitinin anormal proteine bağlanıp işaretleme şekli önemli olup, sadece düz sıralı ubiquitinler PGP 9.5 enzimi tarafından serbest ubiquitinlere ayrılmaktadır. Enzimin bu aktivitesinin fizyolojik önemi tam açık değildir.*

Anahtar Kelimeler: *Protein Gen Ürünü 9.5 (PGP9.5), İnsan beyni, Sentetik substratlar*

Introduction

Ubiquitin carboxyl-terminal hydrolases are a group of thiol proteases [1,2]. Recently, four ubiquitin carboxyl-terminal hydrolase activities, detected by reaction with ubiquitin carboxyl-terminal ethylester, have been observed in calf thymus [3]. These activities have been related to isoenzyme of 30 kDa; the forth with a larger protein of 100-200 kDa. One of the 30 kDa isoenzymes from thymus has been found to be similar to a ubiquitin carboxyl-terminal hydrolase isolated from reticulocytes.

The gene for this hydrolase has been cloned and found to be 54% identical with a neuronal protein called PGP9.5 [4]. The function of the 30 kDa class of ubiquitin carboxyl-terminal hydrolases may be to cleave ubiquitin from side products [5], but they do not act on high-molecular mass ubiquitin-protein conjugates [3]. Ubiquitin carboxyl-terminal hydrolases have been purified from human, bovine and yeast cells. One of the bovine enzymes was found to be identical with a tissue specific protein (hence a marker for neurons) called PGP9.5. This protein was initially detected by high resolution two dimensional electrophoresis [6]. and is one of the most abundant proteins of brain, where its concentration is 50 times greater than in other organs. This suggests that the ubiquitin system is very active in neuronal tissues and that PGP9.5 might be a ubiquitin precursor processing enzyme. It was also thought that the enzyme could prove to be a useful marker in studies of neuronal development and might possibly have clinical applications in detecting damage to the central nervous system [7]. Although PGP9.5 was shown to be essentially a neuron specific protein, it was also found to be present in scattered anterior pituitary cells, thyroid parafollicular cells, pancreatic islets and adrenal medullary cells [8]. The precise function of PGP9.5 as a hydrolase is still not clear.

Primary structure of human PGP9.5, its mRNA and cDNA sequences have been identified [9]. It has a monomer molecular weight of 27 kDa and is a cytoplasmic protein of 212 amino acids [4].

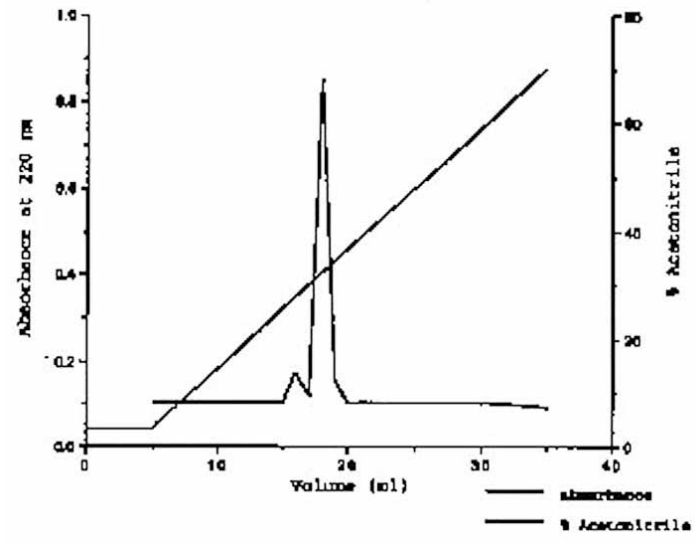

Fig. 1 — HPCL analysis of purified PGP9.5 with C4 reverse phase column. Other conditions were as described in the methods section.

Materials and Methods

Purification of PGP9.5 Enzyme:

PGP9.5 enzyme was purified from human brain obtained after postmortem within 12 hours. Purification procedure was that of Doran et al. [6], and further purification was modification of it [10]. Ubiquitin related peptides were all synthesized on an Applied Biosystems Model 431A Peptide Synthesiser. The synthesis were performed using either t-Boc or Fmoc chemistries according to the protocols established by the manufacturer. Peptide cleavage and deprotection was performed according to procedure described within the manual supplied with the instrument.

High Performance Liquid Chromatography (HPLC); Analytical and Small-Scale Preparative Procedures:

All of the synthetic peptides were assessed for purity by standard-bore reverse phase HPLC using either an Altex Model 110A/334 programmed dual-pump gradient system with a Spectroflow Model 773 variable wavelength detector or an Applied Biosistems 1406A solvent delivery system which 1783A Absorbance Detector Controller, in each case with a chart recorder.

For analysis, peptides were usually dissolved at concentrations of around 1-5 mg/ml in 50% aqueous acetic acid containing a small amount (c.0.5 mg) of solid DTT and left for at least two hours to ensure full reduction of the sulphydryl groups in cysteine residues. Occasionally it was necessary to use 50% aqueous formic acid to obtain a clear solution. Peptide samples were always centrifuged in a MSE Micro Centaur Microfuge at full speed for 5 min prior to injection onto the column.

Separations were effected on a range of standard 4.4 mm bore C4, C8 or C18 reverse phase columns using linear gradients of acetonitrile in aqueous trifluroacetic acid (TFA) at a flow-rate of 1 ml/min. Peptides were detected at 220 nm and, when required, appropriate peaks were collected manually. This small scale procedure is effective for amounts in the 5-500 µg range.

Assay of Purified PGP9.5 with Potential Peptide Substrates:

The assays were performed in 0.1 M potassium phosphate buffer, containing 10 mM DTT and 0.2 mM EDTA, pH 7.2 in a final volume of 1 ml. The concentration of each substrate peptide was adjusted to 0.05 mg/ml. At this concentration of peptide, it was easy to monitor for cleavage by HPLC. Since the level of enzyme activity was not known, two different amounts of PGP9.5 were used (35µg and 140µg purified PGP9.5 Per 50µg peptide) with each substrate. The PGP9.5 that was used in these experiments had been freshly prepared and was used either immediately after isolation by FPLC or after overnight storage at 0°C. Samples were taken at zero time, after 2 hr and after overnight incubation at 37°C. For each reaction a control was prepared without enzyme. After incubation the reaction was stopped by adding acetic acid to final concentration of 30% (v/v).

Results

In order to be certain that this purified protein was indeed PGP9.5. Further characterization was carried out. Since the pro-

posed enzyme assay were to be assessed by reverse phase HPLC, it seemed appropriate to use that procedure to prepare material for amino acid analysis and protein sequence determination. HPLC analyze of PGP9.5 showed a single major sharp peak (Fig. 1). Samples of HPLC purified putative PGP9.5 were applied to slides for automatic amino acid analysis, the results obtained are displayed in Table 1. Where it can be seen that the composition determined is very similar to that expected.

HPLC Purification of Possible Synthetic Peptide Substrates for PGP9.5:

By considering possible functions in the cell for PGP9.5, four potential peptide substrate were chosen for the enzyme assays. Two of the peptides ($UBI_{71-76+1-5}$; part of the linear conjugation of two $UBI_{42-53}K48_{70-76}$ a ubiquitin-related branched peptide) were synthesized by amino acid synthesizer and purified by HPLC.

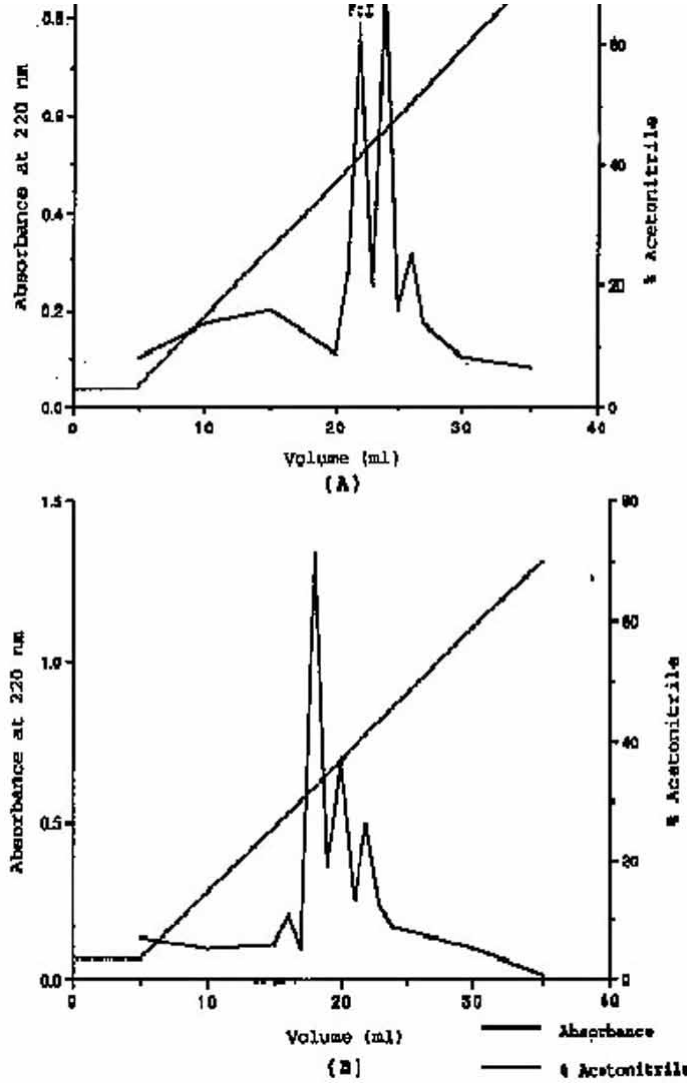

Fig. 2 — HPLC purification of $UBI_{71-76+1-5}$ and $UBI_{42-53}K48_{79-76}$. The peptides ($UBI_{71-76+1-5}$ in A, $UBI_{42-53}K48_{79-76}$ in B) were purified by reverse phase HPLC on a C8 column. Fractions collected manually are labelled FrI and FrII in A and marked with an asteriks in B. Other conditions were as described in the methods section.

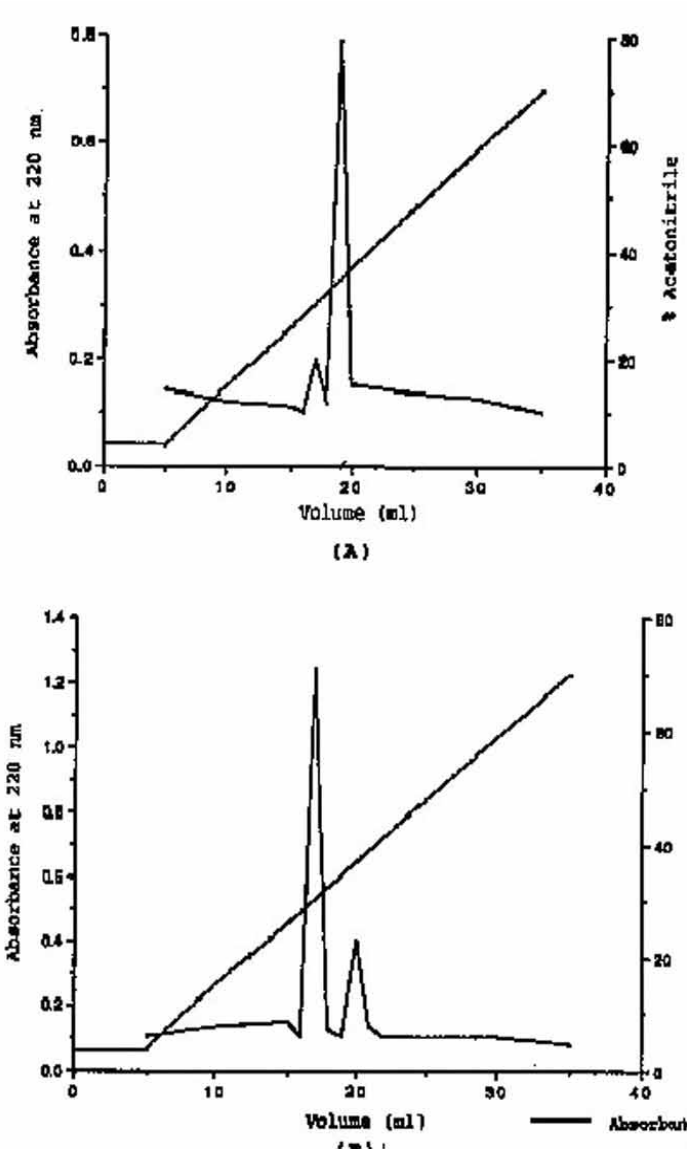

Fig. 3 — HPLC purification of $UBI_{71-76}A52_{1-6}$ and $UBI_{71-76}A80_{1-6}$. The peptides ($UBI_{71-76}A52_{1-6}$ in A, $UBI_{71-76}A80_{1-6}$ in B) were purified by reverse phase HPLC on a C8 column. Fractions collected manually are labelled FrI and FrII in A and marked with an asteriks in B. Other conditions were as described in the methods section.

HPLC purification of them were shown in Fig.2. In the case of $UBI_{71-76+1-5}$ two fraction, designated Fr1 and Fr2 were collected. The latter corresponded to authentic $UBI_{71-76+1-5}$ as shown by sequence determination and appeared to be a mixture of authentic $UBI_{71-76+1-5}$ (possibly with the methionine representing residue 1 oxidized to the sulphoxide form) together with a small proportion (around 25%) of a peptide missing the arginine representing residue 74.

Two additional peptides, representing parts of the two natural ubiquitin fusion proteins [11] were synthesized and designated $UBI_{71-76}A52_{1-6}$ and $UBI_{71-76}A80_{1-6}$; each was an overlap of the carboxyl-terminal six residues of ubiquitin with the amino-terminal six residues of the respective fusion protein. These peptides were also purified by reverse phase HPLC (Fig. 3).

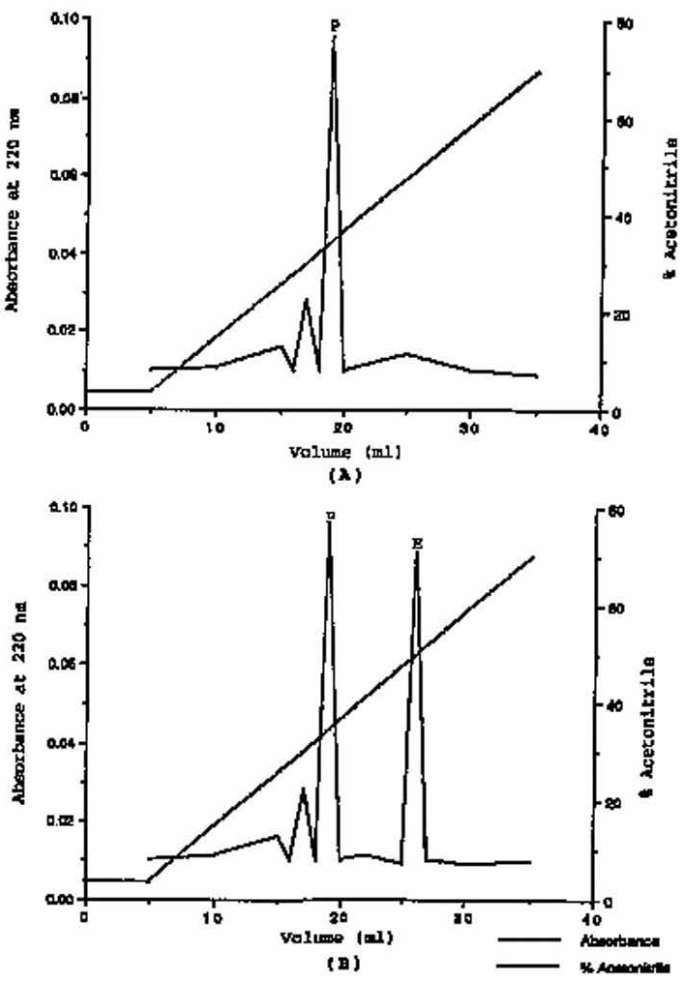

Fig. 4 — Analysis of the reaction of UBI $_{71\text{-}76}$A52$_{1\text{-}6}$ with PGP 9.5. HPLC analysis of the reaction of UBI $_{71\text{-}76}$A52$_{1\text{-}6}$ with PGP 9.5 was performed on a reverse phase (C18 column. A: overnight incubation at 37˚C without PGP 9.5. B: As in A but with PGP 9.5 (140 µg). The peptide peak is marked P, PGP 9.5 as E.

Assessment of Reaction of UBI$_{71\text{-}76}$A52$_{1\text{-}6}$, UBI$_{71\text{-}76}$A80$_{1\text{-}6}$ and UBI$_{42\text{-}53}$K48$_{70\text{-}76}$ with PGP9.5:

The HPLC results in Figs. 4, 5 and 6 indicate that there was no difference between control and enzyme treated samples of these peptides. It would appear that PGP9.5 does not recognize any of the three peptides as a substrate.

Assessment of Reaction of UBI$_{71\text{-}76+1\text{-}5}$ with PGP9.5:

As noted above, from its HPLC profile (Fig. 2) UBI$_{71\text{-}76+1\text{-}5}$ did not appear to be pure. Two peaks of similar size, designated Fr1 and Fr2 had been collected and each was used independently in enzyme assays. There appeared to be some interconversion of Fr1 and Fr2 since both components were present in each purified fraction.

The results of PGP9.5 treatment of both Fr1 and Fr2 showed some differences on the HPLC traces (Fig. 7 and 8). In both fractions, the first peak appeared to show reactivity with the enzyme. There was a large reduction in the height of the peptide peak after two hours and the peak disappeared completely following overnight incubation. In addition, a small additional peak, which was not seen in the control samples, appeared between the peptide and enzyme peaks.

The disappearance of Fr1 following incubation with PGP9.5 was seen in incubations of Fr1 and Fr2 (Figs. 7 and 8 respectively), in each case without any corresponding increase in Fr2 peak height that would be expected if Fr1 was being converted to Fr2. It seems possible that the presence of the oxidized form of methionin allows PGP9.5 to cleave UBI$_{71\text{-}76+1\text{-}5}$. Unfortunately it was not feasible to isolate reasonable amounts of product such that structural analysis could establish that cleavage had occurred. Moreover, it seems improbable that cleavage would be restricted to a non-physiological substrate; the intracellular location of linear ubiquitin synthesis would preclude oxidation of methionin under normal circumstances.

Discussion

In protein degradation, ubiquitin carboxyl-terminal hydrolases disassemble the polyubiquitin chains and cleave the isopeptide bond linking them to the protein substrate. The latter bond also

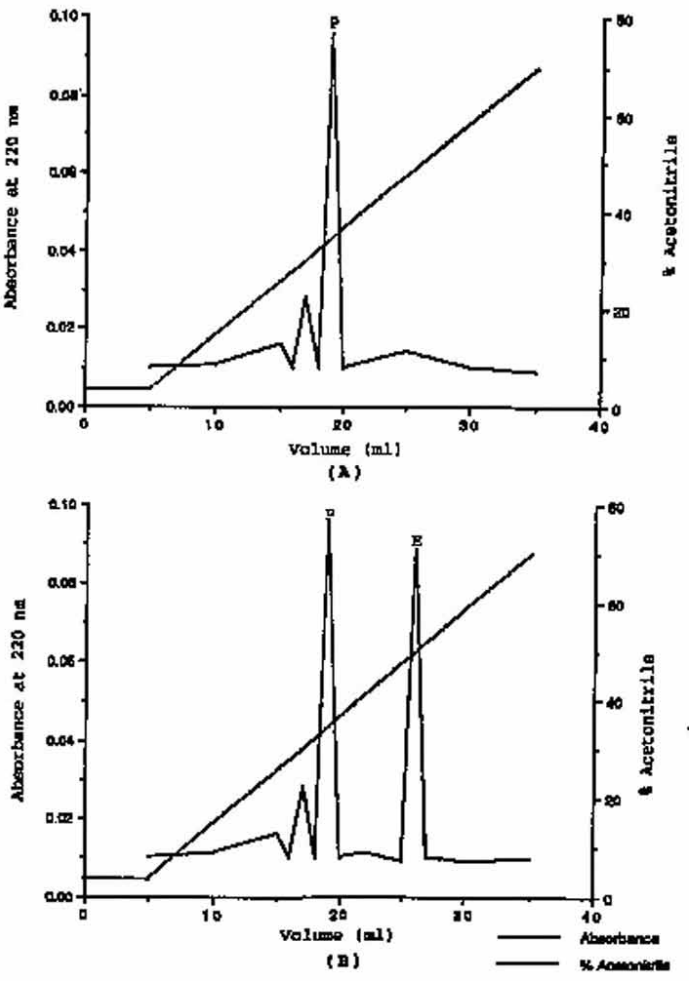

Fig. 5 — Analysis of the Reaction of UBI $_{71\text{-}76}$A80$_{1\text{-}6}$ with PGP 9.5. HPLC analysis of the reaction of UBI $_{71\text{-}76}$A80$_{1\text{-}6}$ with PGP 9.5 was performed on a reverse phase C18 column. A: overnight incubation at 37˚C without PGP 9.5. B: As in A but with PGP 9.5 (140 µg). The peptide peak is marked P, PGP 9.5 as E.

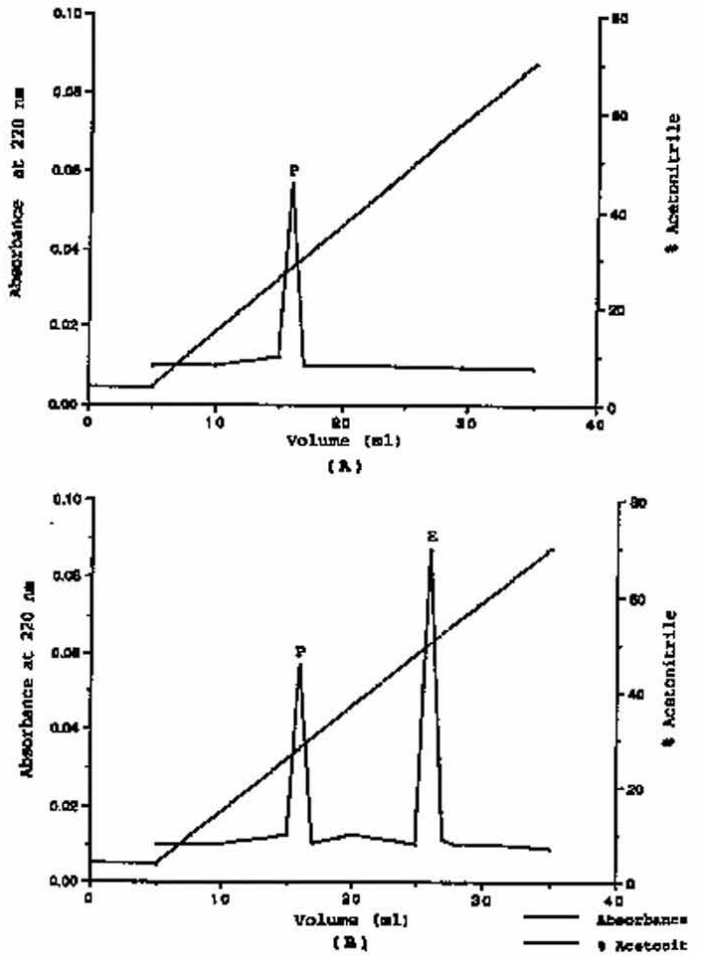

Fig. 6 — Eolysis profiles of various Candida species in human blood SDB (3% glucosAnalysis of the Reaction of UBI $_{42\text{-}53}$A48$_{70\text{-}76}$ with PGP 9.5. HPLC analysis of the reaction of UBI $_{42\text{-}53}$A48$_{70\text{-}76}$ with PGP 9.5 was performed on a reverse phase C8 column. A: overnight incubation at 37˚C C without PGP 9.5. B: As in A but with PGP 9.5 (140 µg). The peptide peak is marked P, PGP 9.5 as E.

occurs in mono-ubiquitinated proteins. Cleavage of linear poly-ubiquitin synthesized from class III genes also needs a ubiquitin carboxyl-terminal hydrolase. It has been found out that PGP9.5 is one of the most abundant proteins of brain. This suggests that the ubiquitin system plays very active role in neuronal tissues. It was also thought that the enzyme might have clinical application in detecting damage to the brain [1,2,4].

That the purification of PGP9.5 was carried out successfully was shown unambiguously by the results obtained by amino acid analysis.

Although PGP9.5 has been shown to have ubiquitin carboxyl-terminal ethyl esterase activity [4,12], detailed information about its substrate approach has been used to investigate potential cleavage of conventional peptide bonds linking ubiquitin to itself in the known branched form of polyubiquitin. No cleavage was apparent under conditions where the enzyme was as fresh as is feasible, albeit prepared from human post-mortem tissue, and at a very high enzyme to substrate ratio. It is possible that peptides might not be suitable for the assay of ubiquitin carboxyl-terminal hydolase activity since the enzymes require a larger substrate; this seems to be unlikely as the specificity of most proteases lies in the peptide sequence of the substrate rather the protein itself. Thus, it seems safe to conclude that PGP9.5 does not cleavage ubiquitin fusion proteins or branched ubiquitin chains.

The situation concerning natural linear chains of ubiquitin as represented by the UBI $_{71\text{-}76+1\text{-}5}$ is less clear. As is argued above, there was some evidence for the cleavage of this peptide, but only when in an alternative form, probably with the methionine oxidized. Since the normal intracellular location of ubiquitin linear conjugates, produced from ubiquitin class III genes, is strongly reducing, there should not be any conversion of methionin to oxidized forms. Thus, the relevance of cleavage of non-physiological forms of poly-ubiquitin, if it does occur, remains unexplained.

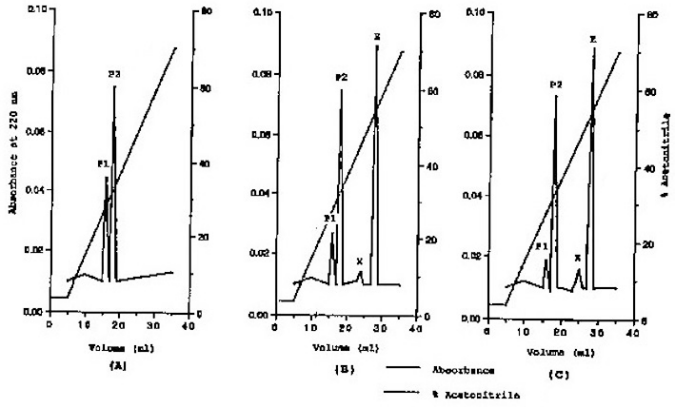

Fig. 7 — HPLC analysis of the reaction of UBI $_{71\text{-}76+1\text{-}5}$ (FrI, see Fig.2.A) with PGP 9.5 was performed on a reverse phase C8 column. A: overnight incubation at 37˚C without PGP 9.5. B: After 2 hours incubations with PGP 9.5 (140 µg). C: As in A but with PGP 9.5 (140 µg). The peptide peaks are marked P1 and P2, PGP 9.5 as E and the extra peak which could not be characterised, as X.

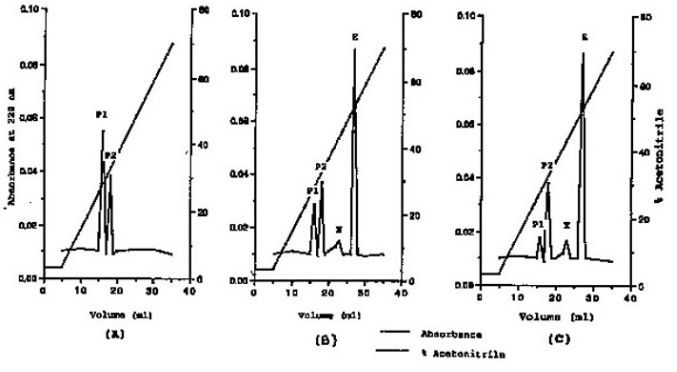

Fig. 8 — HPLC analysis of the reaction of UBI $_{71\text{-}76+1\text{-}5}$ (FrII, see Fig.2.A) with PGP 9.5 was performed on a reverse phase C8 column. A: overnight incubation at 37˚C without PGP 9.5. B: After 2 hours incubations with PGP 9.5 (140 µg). C: As in A but with PGP 9.5 (140 µg). The peptide peaks are marked P1 and P2, PGP 9.5 as E and the extra peak which could not be characterised, as X.

Table 1. Amino Acid Analysis of PGP 9.5. Comparison with the known composition. Cysteine and trytophan are partially or completely destroyed during protein hidrolysis: they have been omitted from the calculation of the estimated number of amino acids present.

Amino acid	Actual number of amino acids from data base	Estimated number of amino acids
D Asp	22	17.1
E Glu	32	33.4
S Ser	11	13.4
G Gly	15	24.0
H His	6	5.8
R Arg	9	9.6
T Thr	6	7.2
A Ala	20	22.9
P Pro	9	10.0
Y Tyr	2	2.4
V Val	17	13.3
M Met	6	2.9
I Ile	7	2.4
L Leu	23	23.1
F Phe	12	13.6
K Lys	16	11.9
C Cys	6	-
W Trp	1	-
Total	220	

Conflict interest statement The authors declare that they have no conflict of interest to the publication of this article.

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